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Analysis of Lipids

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AS A TOOL FOR THE LIPID CHEMIST AND BIOCHEMIST

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I. INTRODUCTION

High-performance liquid chromatography (HPLC) is an analytical tool which came of age in the 1970s. HPLC was readily recognized as an attractive alternative to other methods because of its greater convenience, increased speed, ease of quantification, ease of automation, and ease of preparative scale-ups. Because an estimated 75% of all known chemical compounds (including most underivatized lipids) cannot be analyzed by gas liquid chromatography, HPLC was recognized as a promising alternative chromatographic technique.[1] HPLC methodology was readily developed for analysis of molecules such as proteins, carbohydrates, pharmaceuticals, and pesticides. Until quite recently the number of applications of HPLC to lipid analysis was limited. Some characteristics of lipids appear to have impeded the acceptance of HPLC in the lipid field: (1) the lack of chromophores on many lipids prevented the use of UV-visible detection and (2) the wide range of polarities and solubilities which one encounters with the various classes of lipids made many isocratic analyses impractical. During the last 10 years two major developments have served to open the field of HPLC to lipid analysis. The first was the introduction of sensitive "universal" detectors, such as the flame ionization detector and the evaporative light scattering detector, which could be used in conjunction with HPLC. The second was the introduction of reasonably priced microprocessor-controlled HPLC gradient programmers which could create reproducible ternary and quaternary solvent gradients, especially useful for lipid class separations.

Although other chapters in this volume will present and discuss HPLC methodology for various lipid classes, the goal of the present chapter is to give an overview of the field of HPLC analyses of lipids. We will present many specific examples of published HPLC techniques for the separation and analysis of diverse lipid classes, in order to illustrate the state-of-the-art of the field. In 1982, Aitzetmüller published one of the first reviews on the HPLC of lipids.[1] Volumes I and II of this current series,[2,3] which were published in 1984, contained some references to HPLC but not a chapter entirely devoted to HPLC. In 1986, McCluer et al.[4] published a review on the HPLC of glycosphingolipids and phospholipids. In 1987, Christie published an excellent comprehensive book on the HPLC of lipids.[5] In 1988, Shukla published a review on HPLC of lipids which describes and compares numerous applications.[6] A short review on this topic was also published by Nissen and Kreysel in 1990.[7] Very recently a book has become available on analysis of fats and oils, which contains several up-to-date chapters on HPLC techniques.[8]

II. SAMPLE PREPARATION

A. EXTRACTION OF LIPIDS FOR HPLC ANALYSIS

Although other chapters of this volume will cover the special techniques of lipid extraction, some observations about lipid extractions as they relate to HPLC should be mentioned. There has been good success in using the low-toxicity hexane-isopropanol extraction technique of Hara and Radin[9] for fatty acids[10] and phospholipids.[11] Recently, however, the

following three types of lipids were encountered that are not quantitatively extracted with hexane-isopropanol: (1) hopanoid lipids in bacteria and nitrogen-fixing root nodules;[12] (2) hydroxy and epoxy fatty acids which constitute the monomers of plant cutin;[13] and (3) sesquiterpene phytoalexins, such as capsidiol, phytuberol, and phytuberin which are produced by Solanaceous plants when they are infected by certain fungi and bacteria.[14] In each of these cases the chloroform-methanol extraction technique of Bligh and Dyer[15] proved to be superior to hexane-isopropanol extraction.

B. PREFRACTIONATION OF SAMPLES

For certain types of lipid analyses it may be beneficial to prefractionate the lipid extract before HPLC. Traditionally, open column silicic acid or Florisil chromatography have been used.[5] A convenient type of prefractionation and clean up of crude lipid extracts can also be accomplished using various types of solid phase extraction packings which are commercially available. Such prefractionations of lipid classes by solid phase extraction techniques have been published.[16,17] Preparative TLC has been employed to purify phospholipid classes before separation of molecular species by HPLC.[18]

C. DERIVATIZATION

One of the advantages of HPLC over GLC is that most lipid separations can be achieved without derivatizing the sample. In gas chromatography, the most common reason for derivatizing a sample is to make it more volatile, which is not necessary for HPLC. Reasons for the derivatization of lipid samples before analysis by HPLC are (1) to attach a chromophore or fluorophore to certain constituents to make them easier to detect and (2) to increase the solubility or polarity of some lipids. Chromophore derivatives which have been used in HPLC are phenacyl[19] derivatives of fatty acids and benzoyl derivatives of glycolipids.[4] A useful fluorophore is 9-anthryldiazomethane, which has been reacted with fatty acids,[20] phosphatidic acids,[21] phosphatidylinositol monophosphates,[21] and phosphatidylinositol diphosphates.[21] An example of a type of derivatization which changes the solubility and polarity of a lipid is the acetylation of the hydroxy groups on hopanoids, which decreases their polarity, increases their solubility, and allows them to be separated and quantified by reversed phase HPLC with UV detection.[22]

III. CHROMATOGRAPHY OF LIPIDS BY HPLC

A. CHOICE OF SOLVENTS

In order to achieve a satisfactory HPLC separation, one must choose the right combination of stationary phase (column packing) and mobile phase. Mixed solvents are often able to produce better separations than are possible with a single solvent. Some common HPLC solvents are hexane, chloroform, acetonitrile, isopropanol, methanol, and water (listed in the order of increasing polarity). In reversed phase (partition) chromatography the mobile phase is usually polar (often a mixture of acetonitrile, methanol, and water), the stationary phase is non-polar (such as C_8 or C_{18} bonded to silica gel), and the analytes are eluted in the order of decreasing polarity. In normal-phase (adsorption) chromatography the mobile phase is usually non-polar (such as hexane-isopropanol or chloroform-methanol), the stationary phase is polar (such as silica gel), and the components are eluted in the order of increasing polarity.

B. ISOCRATIC vs. SOLVENT GRADIENT PROGRAMS

Many separations, especially those of complex mixtures of lipids, require the use of solvent gradient programs to achieve optimal separation and resolution of its components.

A useful HPLC accessory is an HPLC gradient programmer, which is a mixing system that uses a microprocessor to form a solvent gradient from two, three, or even four solvents differing in polarity. The major advantage of solvent gradient programs over isocratic separations is that they permit the separation of lipid classes having very diverse polarities in one run. Solvent gradient programs usually require more time than isocratic analyses.

C. CHOICE OF COLUMNS

Because HPLC columns must withstand very high internal pressures (up to 6000 psi), most of the columns and tubings used are made of stainless steel. For analytical HPLC, column diameters usually range from 2 to 5 mm and column lengths from 5 to 25 cm. For preparative HPLC, column diameters range from about 1 cm to 1 m. In general, the smaller the column diameter, the lower the flow rate of solvent (which can both conserve solvents and increase the sensitivity of an analytical separation). Good lipid separations can be achieved with cartridge (3 × 100 mm) columns[14,23,24] which are available with a wide range of packing materials. These glass cartridges fit into a stainless steel holder. Because they are less costly than conventional columns, they can usually be used without a guard column. A guard column is a short column which is placed before an analytical HPLC column to protect it from contamination. Omission of the guard column has been found to increase the resolution of some lipid separations.[25] The following types of phases are routinely used in HPLC of lipids: (1) silica gel, cyanopropyl, aminopropyl, diol, silver ion (for normal phase separations); (2) C18, C8, phenyl (for reversed phase separations); (3) DEAE, TEAE (for ion exchange separations); (4) gel permeation (molecular sieve) columns; and (5) chiral phase columns. Examples of lipid separations with each of these types of columns will be described in a later section.

D. DETECTORS

1. Ultraviolet (UV) Detectors

These instruments are the most common type of detector found in the analytical laboratory. Most instruments are able to measure absorbance in the range of wavelengths from 190 to about 750 nm and should more accurately be referred to as UV-visible detectors. Although most modern units utilize monochromators that allow the selection of virtually any wavelength in the UV-visible range, less expensive units use filters to select a particular wavelength. Both types of instruments are usually quite sensitive to a given analyte if it possesses a UV or visible chromophore. Another, more costly type of ultraviolet detector which has become available is the diode array UV-visible detector. Instead of monitoring the absorbance at a single preselected wavelength, the diode array detector utilizes electronic technology to obtain a series of spectra, over a range of wavelengths, at about 100 μs intervals. Because some lipids exhibit little or no UV absorption, the usefulness of UV detectors for their analysis is limited. Only lipids which have conjugated double bonds or aromatic rings exhibit strong absorption in the UV range. However, many acyl lipids (especially those with one or more carbon-carbon double bonds) show measurable absorbance in the range of 200 to 220 nm. Solvents such as chloroform and methylene chloride exhibit strong absorbance in the 200 to 220 nm region and therefore cannot be used for separations that are monitored with UV detectors. Most other common organic solvents have little or no absorbance above 200 nm and can be used with this type of detector. UV detectors can be used with various solvent gradient programs as long as they are composed of solvents which are non-absorbing at the wavelengths used.

2. Refractive Index (RI) Detectors

These instruments are probably the second most popular type of detector found in the analytical laboratory. They measure the difference in refractive index between the pure

solvent and the solution of constituents of the analyte(s). Although the original RI detectors were rather insensitive, they were utilized for some of the first studies on the HPLC of lipids. The improved units on the market are considerably more sensitive and hence useful for lipid analysis. Precautions must be taken to ensure that the temperature of the column and tubing, and the composition of the mobile phase remain constant. Also, the sample must be dissolved in the solvent used as the mobile phase. The minimum limit of detection with modern RI detectors is about 0.1 to 1 μg. As a rule, the relationship between mass and peak area is linear. A recent example of a study utilizing an RI detector involved the analysis of phosphatidylcholine.[26]

3. Evaporative Light Scattering Detectors (ELSD)

These detectors are sometimes referred to as "mass" detectors and presently are produced by three manufacturers: Applied Chromatography Systems (ACS) in the U.K., Cunow in France, and Varex in the U.S. The way these detectors operate is by rapidly evaporating the mobile phase by means of a nebulizer. This is achieved by mixing and spraying the eluent stream with a large volume of either nitrogen or air. While the more volatile solvent is evaporated, the solute remains as droplets which are directed through a source of light or laser light where the degree of scattering of the light is proportional to the mass of the solute. ELSD have proven to be highly useful for the HPLC of lipids since they can be used with most common organic solvents, and under ideal conditions, with high levels of water in the mobile phase. These detectors cannot, however, be used with inorganic acids, bases, or salts. Because ELSD are quite insensitive to mobile phase composition, they can be used with gradient programs. In fact, essentially flat baselines have been reported using such detectors with solvent gradient programs which span a very wide range of solvent polarities.[5,24,27]

Some other advantages of ELSD are their ease of use, low maintenance, and the fact that temperature control is not required. There are, however, three disadvantages which ought to be considered. The first is that these units use large volumes of nebulizer gas, up to 500 ml/min. The second is that the ratio of mass to peak area is not always a linear function. It has been found that for most lipids this relationship is linear in the range of about 10 to 200 μg, but below 10 μg it is a parabolic. This situation can be rectified by running a series of standard curves. The third disadvantage has to do with the volatility of the analyte; if the analyte is even slightly volatile, it will be evaporated with the mobile phase solvents and will either remain undetected or produce only a weak signal. Even palmitic acid is volatilized in the ELSD and therefore the temperature of the detector has to be lowered in order to detect fatty acids with chain-lengths $\leq$ 16 carbon atoms.

4. Flame Ionization Detectors (FID)

The first flame ionization detector for HPLC was marketed for a short time by Pye Unicam in the mid 1970s.[28] A similar "home-made" instrument was constructed by Privett et al. and successfully used in lipid analysis.[29-31] Flame ionization detectors for HPLC are similar to their popular counterparts in gas chromatography, in that the analyte is burned in a flame and the resulting carbon ions are measured by electrodes placed across the flame. Manufacturers of flame ionization detectors have developed two strategies for removing the mobile phase solvents before the solute reaches the flame. In the Tremetrics instrument (formally called Tracor) the solvent stream is sprayed through a small orifice onto a porous revolving quartz belt. The solvents of the mobile phase are evaporated from the belt by heating *in vacuo*. After the solvents have been removed the belt passes through a hydrogen-air flame and the deposited solutes are ionized and detected.

Unlike the ELSD, the baselines obtained with the FID are not always flat. Specifically, when using gradient programs which contain polar solvents such as methanol or isopropanol, the baseline increases with increasing levels of these solvents.[23,24] Even low levels of acetic or formic acids produce very high background signals with an FID. Like the ELSD, these detectors cannot be used with solvents containing inorganic acids, bases, or salts. An advantage of the FID is that the ratio of mass to peak area is linear in the range of 1 to 200 μg.[23,24] When carrying out isocratic elution with a mobile phase of hexane-iropropanol, the linear range of the FID can be extended to 0.1 to 30 μg.[14] A disadvantage of the FID over the ELSD is that the current units require a considerable amount of non-routine maintenance. Another disadvantage is that volatile analytes (as described above for the ELSD) will also not be accurately quantified by the FID.

5. Fluorescence Detectors

These detectors consist of the same basic optics and electronics as a UV detector, but unlike the UV detector they contain two monochromators, one for the excitation wavelength and one for the emission wavelength. In order to measure fluorescence in the HPLC flow cell, the excitation monochromator is placed at a right angle to the emission monochromator. Fluorescence detectors can be useful for the analysis of the few lipids that exhibit fluorescence, such as chlorophyll and other pigments. In addition, they can be useful for the detection of lipids which have been derivatized by adding a fluorophore.[20,21]

6. Radioisotope Detectors

Numerous biochemical studies have been accomplished by using labeled compounds and measuring their metabolites using radioisotope detectors.[32,33] These instruments provide an extremely sensitive technique for the detection of lipids that are labeled with radioisotopes. They can be especially useful for quantifying lipids which are labeled with ^{3}H, ^{14}C, ^{35}S, or ^{32}P.

IV. COMPARISON OF HPLC METHODS

A. HPLC METHODS FOR THE SIMULTANEOUS ANALYSIS OF BOTH NON-POLAR AND POLAR LIPID CLASSES

Because of the great diversity of polarities which are encountered among the various lipid classes, the task of developing an HPLC analysis which could perform a simultaneous separation of such a mixture of components was not an easy one. Two laboratories developed separate approaches to this problem during the 1970s. Using an HPLC system with a Pye Unicam flame ionization detector, Kuichi et al.[28] worked out a method which could separate triacylglycerols (TAG), sterols (ST), free fatty acids (FFA), monoacylglycerols, and phospholipids. The analysis was performed on a silica gel column with a mobile phase that began with hexane-chloroform and included a linear binary gradient of 0 to 70% ethanol. Using a flame ionization detector Phillips et al.[29-31] developed a method which allowed separation of 15 lipid classes that ranged in polarity from retinyl esters to lysophosphatidylcholines (lyso-PC). The stationary phase was silica gel which had been treated with ammonium hydroxide. The column was eluted first with petroleum ether-methylene chloride, and then with a linear gradient of ammonium hydroxide. Later, Erdahl and Privett[34] coupled the same lipid class separation system to a chemical ionization mass spectrometer. Maxwell et al.[35] developed a similar method (utilizing an ammonium hydroxide-treated silica gel column and a gradient system of hexane-methylene chloride-chloroform-methanol-ammonium hydroxide) which employed the Tracor flame ionization detector to record diverse lipid classes. Although the technique worked well with lipid standards the authors did not report analyses of any natural lipid extracts.

In a separate approach, Christie developed a rapid HPLC system which employed a silica gel column and an ELSD.[27] The method involved a ternary gradient system which successfully separated very diverse lipid classes from a complex lipid extract isolated from animal tissues. The three solvent mixtures in this gradient system were isooctane-tetrahydrofuran (99:1, v/v), isopropanol-chloroform (4:1, v/v), and isopropanol-water (1:1, v/v). In a subsequent paper,[36] Christie reported two improvements to this gradient system; isooctane was replaced by hexane to reduce the maximum operating pressure, and the water was buffered with 0.5 m*M* serine, adjusted to pH 7.5 with triethylamine, to sharpen the resolution of the minor acidic components.

Our group recently published a similar ternary gradient method to analyze lipid classes from plant tissues.[23] A silica column, a ternary gradient of hexane-isopropanol-water, and an FID were employed. The gradient program was more gradual than that of Christie[27] in order to separate the plant glycolipids. Also, because it did not contain chloroform, a UV detector could be used either in tandem with the FID or alone. Christie had reported that the inclusion of chloroform in his system helped to sepharate phosphatidylcholines (PC) and sphingomyelins (SM),[27] however, since plants do not seem to contain SM, there is no need to include it in our system. In a subsequent study,[24] using the same gradient system with an ELSD three major differences were observed: (1) there was less baseline noise with the ELSD than with the FID; (2) the ELSD was slightly more sensitive than the FID; and (3) the mass to signal ratio was less linear with the ELSD than with the FID. This HPLC-FID system was recently employed to analyze the bacterial lipids in symbiotic root nodules from nitrogen-fixing trees;[12] the results with the flame ionization detector indicated that the most abundant lipid class in these root nodules and in *Frankia,* the symbiotic bacterium found in these nodules, is bacteriohopanetetrol (Figure 1A). For comparison, the chromatogram of the same extract recorded with a standard UV detector is shown in Figure 1B. The most striking difference between the two chromatograms is that although bacteriohopanetetrol (a saturated pentacyclic terpene) is the most abundant lipid component (comprising about 40% of the total lipid mass), it is completely undetected by the UV detector. These data present a convincing argument for the usefulness of the new types of universal HPLC detectors (FID and ELSD) for lipid class analysis.

Other laboratories have recently reported additional variations of Christie's gradient system for lipid class analysis.[37,38] In the first variation, Lutzke and Braughler[37] employed an ELSD and the solvent mixtures reported by Christie,[36] with a slightly different gradient program. The latter authors reported minimum limits of detection of 50 ng for neutral lipids and 200 ng for most phospholipids, with excellent reproducibility. Redden and Huang,[38] also using conditions almost identical to those of Christie,[27] automated the system and incorporated a computer to calculate the mass of each peak via a quadratic equation.

In the examples given in this section, non-polar and polar lipids were simultaneously analyzed in a single injection. Recently, Hamilton and Comai reported a way to obtain a complete lipid class analysis by performing a series of three separate normal phase isocratic runs for each lipid extract.[39] The method employs a silica gel column and a UV detector. The first two isocratic mixtures contain hexane-methyl *tert*-butyl ether-acetic acid in different proportions, and the third one contains methyl *tert*-butyl ether-methanol-aqueous ammonium acetate. The non-polar lipids are separated in the first run, the free fatty acids in the second run, and the phospholipids in the third run. Since each separation takes about 10 min, the method requires only 30 min for an analysis of a total lipid extract. Thus, this technique is more laborious than the above types of gradient fractionations, but the total time for each sample could be equal to or even less than the time required for comparable gradient analysis. Using bonded phase columns (which are more durable than silica gel columns) and mixtures

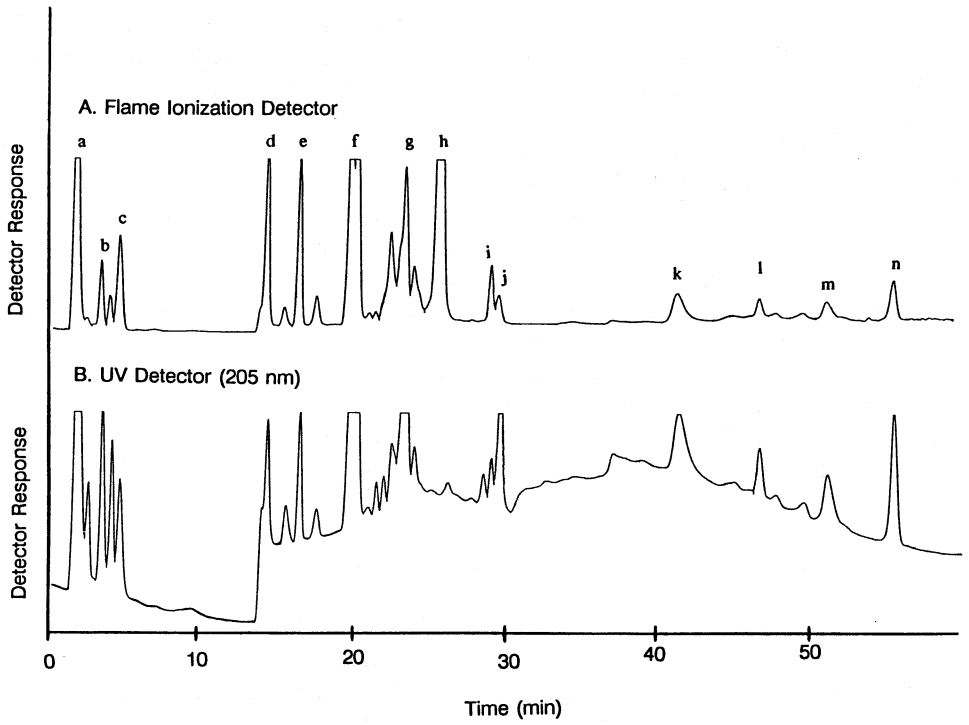

FIGURE 1. A comparison of a lipid class separation of an extract from the nitrogen-fixing nodules of *Alnus* roots, monitored with a flame ionization detector and a UV detector. Extraction and HPLC chromatographic conditions were as previously described[12] with the addition of a UV detector in tandem with the flame ionization detector. The peaks were identified as: (a) hydrocarbons; (b) sterol esters; (c) triacylglycerols; (d) sterols; (e) free fatty acids; (f) hopanoid esters; (g) acylated sterol glycosides; (h) bacteriohopanetetrol; (i) glucocerebrosides; (j) monogalactosyldiacylglycerols; (k) digalactosyldiacylglycerols; (l) phosphatidylethanolamines; (m) phosphatidylinositols; (n) phosphatidylcholines. It should be noted that although bacteriohopanetetrol (h) is one of the most abundant lipids in these extracts,[12] it is not detected by the UV detector.

of hexane-isopropanol-water we have recently developed similar isocratic procedures for the separation of many diverse types of lipid classes (Table 1). Since these solvent mixtures are noncorrosive and are compatible with all common HPLC detectors, it is believed these types of separations may find many applications. As mentioned earlier, minimum limits of detection of the Tracor flame ionization detector are much lower using isocratic conditions, such as reported in Table 1, than with solvent gradient programs.[23]

B. HPLC METHODS FOR THE ANALYSIS OF NON-POLAR ACYL LIPIDS

Hamilton and Comai[40] worked out an isocratic, normal phase separation of cholesterol esters, triacylglycerols, free fatty acids, and sterols. They used a silica gel column and hexane-*n*-butyl chloride-acetonitrile-acetic acid (90:10:1.5:0.01, v/v). Palmer et al.,[41] using a cyanopropyl column and a UV detector, reported the separation of cholesterol esters (this fraction was eluted at the solvent front and also included retinyl esters and dolichyl esters), triacylglycerols, vitamin E, ubiquinone, dolichol, and cholesterol in only about 12 min with 0.05% isopropanol in hexane. They similarly reported the separation of cholesterol, retinol, diacylglycerols, and monoacylglycerols, in about 8 min with 0.75% isopropanol in hexane. These separations are comparable to those we have obtained under similar conditions (Table 1). Using an isocratic reversed phase system with acetone-acetonitrile (1:1, v/v), Veeraragavan[42]

of a chromophore or fluorophore to the fatty acids to enhance their detection, as described in a previous section. Another chapter in this book, authored by Sebedio, covers the topic of HPLC of fatty acids in detail. In addition, other authors have provided thorough descriptions and comparisons of the various techniques.[5,6] A sensitive HPLC method for the analysis of underivatized fatty acids using one of the newer universal HPLC detectors remains to be developed. More specialized HPLC procedures have been developed for the analysis of hydroxy fatty acids,[46] cyclopentenyl fatty acids,[47] epoxy fatty acids,[13] and keto fatty acids.[13]

In addition to the above normal phase and reversed phase methods for HPLC of non-polar lipids, size exclusion (gel filtration, gel permeation, and molecular sieve) HPLC has been used to separate triacylglycerols and other non-polar lipids. It is an especially useful method for polymerized lipids, such as those generated during deep fat frying.[5] In a recent report, a size exclusion column was used with a refractive index detector to analyze monomers, dimers, and trimers of fatty acids in thermally oxidized fats and oils.[48] In another study, size exclusion HPLC in combination with an ACS evaporative light-scattering detector was used to analyze polymers in autoxidized marine oils.[49]

A significant advance in HPLC of lipids is the development of methods to analyze stereoisomers of triacylglycerols and other lipids by chiral HPLC.[50,51] This topic was recently reviewed by Takagi[51] and is covered in Chapter 10 of this volume.

C. HPLC METHODS FOR THE ANALYSIS OF POLAR ACYL LIPIDS

Several of the methods described in the previous discussion on HPLC techniques for the simultaneous analysis of non-polar and polar acyl lipids provide excellent separation and quantification of most of the common polar acyl lipids (glycolipids, phospholipids, and sphingolipids). For investigators who have ternary solvent gradient HPLC systems and an ELSD or FID available, the technique of Christie,[27,36] or any of its variations[23,24,37,38] would provide quantitative analyses of most polar lipids as well as non-polar lipids.

The separation of phospholipid classes has been performed by TLC for many years. In the mid-1970s three groups have developed techniques which attempted to accomplish such analyses by HPLC. Jungalwala et al.[52] published the first HPLC separation of phospholipids using a silica gel column and a gradient of acetonitrile-methanol-water. Others reported improvements of this system by adding ammonium hydroxide[4] or phosphoric acid.[53] Geurts Van Kessel et al.[54] published an HPLC method for the separation of phospholipids using a silica gel column and a binary gradient of hexane-isopropanol-water. Others reported improved separations by adding strong acids[55] or buffers[56] to this system. Another approach to the HPLC of phospholipids was that of Kuichi et al.[57] They used a Pye Unicam flame ionization detector, an amino column, and a solvent system consisting of chloroform-methanol-water, to separate the phospholipid classes.

A disadvantage of the hexane-isopropanol-water system is that it does not adequately separate PC and sphingomyelin. To overcome this problem Juaneda and Rocquelin[58] worked out a two-step HPLC procedure in which the hexane-isopropanol-water system was followed by an acetonitrile-methanol-water system to separate PC and sphingomyelin.

Since this topic was last reviewed[5,6] several investigators have reported "new and improved" methods for separation and analysis of phospholipid classes.[60-69] Upon close examination it appears that most of these recently reported procedures[60-69] find their roots in the above-mentioned techniques. Some aspects of the analysis of phospholipids are covered in Chapter 16 of this volume.

Several specialized techniques have been published for the simultaneous analysis of plant galactolipids and phospholipids. Demandre et al.[70] reported the separation of monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and the common

TABLE 1
Separation of Lipid Classes with Bonded Phase Columns and Several Isocratic Mixtures of Hexane-Tetrahydrofuran-Isopropanol-Water

Lipid Class	Column	Mobile phase composition (v/v) (%)				
		Hexane	**100**	**99**	**97**	**90**	**40**
		THF	**0**	**1**	**0**	**0**	**0**
		Isopropanol	**0**	**0**	**3**	**10**	**57**
		Water	**0**	**0**	**0**	**0**	**3**
		Retention time (min)					
Sterol esters	CN		2.5	1.1[a]			
Triacylglycerols (TAG)	CN			2.8			
Fatty acids (FFA)	CN			7.0			
Sterols (ST)	CN		22.0	9.8	1.1[a]	1.1[a]	1.1[a]
Rishitin	CN				4.5		
Lubimin	CN				7.1		
Capsidiol	CN				11.5		
Acylated sterol glycosides (ASG)	CN				18.6	2.5	
Bacteriohopanetetrol	CN				21.0		
Sterol glycosides (SG)	CN					5.2	
Glucocerebrosides	CN					6.9	
Monogalactosyldiacylglycerols (MGDG)	CN					7.2	
Phosphatidylethanolamines (PE)	Diol						2.3
Phosphatidylcholines (PC)	Diol						3.8
Sphingomyelins (SM)	Diol						4.5
Lysophosphatidylcholines (lyso-PC)	Diol						10.8

Note: The columns used were Spherisorb CN (5 μ), and LiChrosorb Diol (5 μ), Chromsep (Chrompack Inc.) cartridges (3 × 100 mm), and the solvent flow rate was 0.5 ml/min.

[a] This component elutes in the void volume in this mobile phase, possibly along with other less polar components, and for this reason may not be pure.

reported successful separation of free fatty acids, tri-, di-, and monoacylglycerols in about 20 min.

Considering the great nutritional importance which has been attributed to the fatty acid composition of vegetable oils, it is not surprising that numerous HPLC procedures have been developed for the analysis of molecular species of triacylglycerols. Several reversed phase chromatographic methods for the separation and quantification of molecular species of triacylglycerols have been compared in a recent review.[6] In a later report, the separation and quantification of molecular species of triacylglycerols was accomplished on a C_{18} column with a binary gradient of methylene chloride-acetonitrile and an ELSD.[43] Laasko and Christie[44] reported a two-step HPLC technique for separating molecular species of triacylglycerols. This technique has been shown to be especially useful for the analysis of oils that contain numerous molecular species of triacylglycerols, such as fish oils. In the first step, a silver ion column is used to separate triacylglycerols according to their total number of double bonds; the second step involves reversed phase separation on a standard C_{18} column.

Although less intensely studied, methods for analysis of the molecular species of cholesterol esters are of interest to the medical community, and recently a simple isocratic method was developed to separate cholesterol and its common esters.[45] It utilized a C_{18} column and acetonitrile-isopropanol (1:1, v/v) as solvent.

Fatty acids are routinely analyzed by converting them to methyl esters and analyzing these by gas chromatography. However, several methods have been developed to analyze other fatty acid derivatives by HPLC. Most of these techniques have required the attachment

phospholipid classes on a silica gel column with a binary gradient of hexane-isopropanol-water. Heemskirk et al.[71] described similar separations with the same solvents plus methanol on an amino column. Evans et al.[72] achieved better separations using a silica gel column and a binary gradient of hexane-isopropanol 2.8 m*M* ammonium acetate. Christie and Morrison[73] worked out a similar technique for the separation of cereal glycolipids.

Several other specialized techniques for the separation and/or analysis of individual phospholipid classes have been published in recent years. These include methods for the analysis of PC[26,74] and phosphatidic acids (PA).[75] Two techniques have been developed for the analysis of phosphoinositides (phosphatidylinositol monophosphate and phosphatidylinositol diphosphate), one requires derivatization[21] to attach a chromophore, and the other is a reversed phase technique.[76] Pagano's group has published some elegant studies of phospholipid metabolism using a phospholipid analogue which contains a cyclic fatty acid that fluoresces, permitting one to study the cell biology of lipid metabolism via fluorescence microscopy.[77] The same group has developed an HPLC method to analyze fluorescently labeled phospholipids.[78]

For many types of enzymatic and metabolic studies, biochemists may require the purification of classes of polar lipids. Therefore several laboratories have developed procedures for the preparative HPLC of phospholipids.[79-81]

Not long after the first HPLC methods had been developed for the successful separation of classes of polar lipids, procedures became available for the separation of molecular species of various phospholipids and glycolipids. Arvidson[82] published the first method for the separation of molecular species of PC. It employed an early type of reversed phase column (the stationary bonded phase was a mixture of C_{11} to C_{14} chain lengths), a mobile phase of methanol-water, and a refractive index detector. Most subsequent methods have also employed reverse phase columns, especially C_{18} phases. In order to accurately quantify individual peaks of molecular species, some workers have analyzed "intact" phospholipids using universal detectors (ELSD or FID),[18] while others have attached UV chromophores[83] or fluorophores.[84] Another approach to quantification of molecular species has been to use dual wavelength UV detection.[85] A specialized ion-pair HPLC technique has recently been developed to separate molecular species of phosphatidylinositols.[86] Similar reversed phase techniques have been published for the separation of molecular species of intact plant galactolipids[33] and intact sphingomyelins.[87] In addition to the above analytical HPLC techniques two recent publications describe a preparative HPLC procedure for purifying molecular species of phosphatidylcholine[88] and some plant galactolipids.[89] Excellent detailed discussions of the HPLC of sphingolipids are contained in previously published reviews.[4,5]

D. HPLC METHODS FOR THE ANALYSIS OF NONACYL LIPIDS

In the following section recent HPLC techniques for the separation of sterols and other steroids, terpenes, tocopherols, chlorophylls, carotenoids, flavanoids, and coumarins are presented. In this chapter, nonacyl lipids are defined as those chloroform-soluble molecules which either do not contain fatty acyl components or are not derived from fatty acids.

The separation of sterols and terpenes present in complex natural mixtures, such as oils[90] and seeds of *Chenopodium quinoa*,[91,92] has been achieved by HPLC. Other methods have been used by Farines et al.[93] for the preliminary separation of sterols and for the discovery of new sterols and triterpene alcohols. Diastereoisomers of terpenic alcohols have been studied by Italia et al.[94] using cyclodextrin bonded phase columns and cyclodextrin mobile phase modifiers.

Gossypol, a toxic triterpene aldehyde found in various parts of the cotton plant[95] and in processed cotton oil,[96] has been analyzed by HPLC. The HPLC method of Hron et al.[97]

gave results comparable to that of the official AOCS method for gossypol measurement. Pietta et al.,[98] succeeded in separating terpenes from *Ginkgo biloba,* using a C_{18} column both on an analytical and a semi-preparative scale. West et al.[99] compared GC and HPLC methods for the analysis of pentacyclic triterpenes. Kanazawa et al.[100,101] successfully quantified various tetra- and pentacyclic triterpenes from the roots of *Panax ginseng*. Triterpenoids from *Ganoderma lucidum* have been separated by normal phase[102,103] and reversed phase[104] HPLC techniques. HPLC techniques[105] have also been developed for taxonomic studies of terpenoids. Hopanoids, which are pentacyclic triterpenes, recently detected in many species of bacteria, have been measured using reversed phase HPLC techniques.[22,106] Reference is also made to the study of hopanoids in *Frankia*[12] as previously discussed (see Figure 1). Morin et al.[107] reviewed analytical and preparative HPLC separations of terpenoids, and presented a semi-preparative HPLC method for the purification of terpenoids from oils. Orsini and Verotta[108] have studied various reversed phase methods for the separation of triterpenoid glycosides in *Passiflora quadrangularis* L. Domon et al.[109] succeeded in separating oleanane saponins on C_8 and Diol columns.

HPLC techniques have been used to analyze the sterol composition of various biological materials. Trost[110] has extensively used both analytical and semi-preparative HPLC columns to separate sterols and other non-polar materials from oils. Reversed phase and normal phase methods have been used to quantify cholesterol (see Reference 111 for a review), fungal sterols,[112] and dehydrocholesterol in human skin[113] and in human lipoprotein.[114] HPLC has been used by Van de Bovenkamp et al.[115] for the quantification of oxysterols in eggs and mixed diets. HPLC analyses of sterols and tocopherols have been applied to plasma,[116] human milk lipids[117] and foods.[111] Other sophisticated techniques, such as LC-GC have been useful for the analysis of sterols in citrus oil[118] and for the analysis of sterols and wax esters in olive oils.[119] Warner and Mounts[120] have recently published a procedure for the analysis of phytosterols and tocopherols in vegetable oils by reversed phase HPLC-ELSD. Maerker et al.[121] have reported a technique for the separation and quantification of cholesterol oxidation products by normal phase HPLC with FID.

The analysis of steroid hormones in various biological fluids has been achieved using both reversed phase and normal phase HPLC methods (see Reference 122 for a review and Reference 123 for a comparative study of these methods). Noggle et al.[124] developed isocratic HPLC methods to study anabolic 17-hydroxy steroids, controlled substances in the U.S. Medina and Sherman[125] published a normal phase HPLC method for the separation of anabolic oestrogens. Ahmed and Riaz[126] developed a reversed phase system for the separation of various classes of steroids. The combination of HPLC and radioimmunoassay is becoming a popular technique for the detection of steroids in urine,[127] meat samples,[128] and serum.[129] Several applications of HPLC for the analysis of polyhydroxysteroids found in plants and invertebrates have been reported.[130-132]

Normal phase techniques were reported for the quantitative analysis of tocopherols in various vegetable products.[134-137] Reversed phase HPLC was developed to analyze phenolic antioxidants, tocopherols, and triacylglycerols in sunflower oil,[138] cod liver oil,[139] and in human buccal mucosal cells.[140] Aminopropyl bonded phase columns were recently used for the quantification of tocopherols in milk[117] as well as in frying fats and oils.[141] Cyanopropyl phases were employed by Shukla[142] for a rapid determination of tocopherols in various vegetable oils. A technique mentioned above for the analysis of phytosterols in vegetable oils is also useful for the analysis of tocopherols in such oils.[120]

Several applications of reversed phase HPLC have been published for the analysis of plant pigments (chlorophylls and carotenoids) in phytoplankton,[143] various vegetable tissues,[144] and *Nicotiana tabacum* leaves.[145] Quantitative analyses of chlorophylls and their derivatives from plant tissues[146,147] and in oils[148,149] have been achieved by both normal

phase and reversed phase chromatography. Although both UV-visible and fluorescence detectors have been employed for various HPLC analyses of pigments, it appears that quantitative comparisons of the two detectors have never been published.

HPLC analyses of carotenoids in seed oils,[150] green pepper[151] and other vegetables,[152,153] and in plant chloroplasts[154] have been reported. Cyclodextrin bonded phase columns were used to separate various isomers of carotenoids.[155] HPLC has also been used to analyze the transformation of various isomers of carotenoids during processes, such as cooking, lyophilization, and freezing.[156,157] Recently, a preparative HPLC method for carotenoids has been reported.[158]

Several reversed phase and normal phase HPLC procedures have been developed for the analysis of flavanoids in fruits and vegetables. Van de Casteele et al.[159] applied reversed phase HPLC to separate 141 flavanoids which ranged in polarity from aglycones to triglycoside flavanoids. HPLC techniques for the analysis of flavanoids in various plant tissues have been reported.[160-163]

Thompson and Brown[164] extensively studied separations of 67 coumarins of higher plants by normal phase and reversed phase HPLC. Van de Casteele et al.[165] separated various coumarins by reversed phase chromatography with various isocratic and linear gradient systems.

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